Immune response to an endotoxin challenge involves multiple immune parameters and is consistent among the annual-cycle stages of a free-living temperate zone bird

Arne Hegemann[1*], Kevin D. Matson[1], Maaike A. Versteegh[1], Auxiliadora Villegas [2] & B. Irene Tieleman[1]

[1]Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, P.O. Box 11103, 9700 CC Groningen, The Netherlands

[2] Conservation Biology Research Group, Faculty of Science, University of Extremadura, Avda. Elvas s/n 06071 Badajoz, Spain

* Corresponding author, e-mail: a.hegemann@rug.nl.

Short title: Immune response throughout annual cycle

Abstract

Trade-offs between immune function and other physiological and behavioral processes are central in ecoimmunology, but one important problem is how to distinguish a reallocation of resources away from the immune system from a reallocation or redistribution within the immune system. While variation in baseline values of individual immune parameters is well established, studies in wild animals on multiple parameters during an immune response are lacking. It also remains to be tested if and how immune responses correlate with baseline values that vary e.g. over the course of an annual cycle. We studied immunological responses to an endotoxin challenge in skylarks (*Alauda arvensis)*, a partial migrant bird breeding in temperate zones. We compared birds injected with the endotoxin LPS with un-injected controls, characterizing immunological responses with leukocyte profiles, titres of lytic enzymes and natural antibodies, and concentrations of haptoglobin and heat shock proteins. We did this in five annual-cycle stages to test if the response varied throughout the year. The endotoxin challenge affected 6 of 10 measured parameters. Lysis titers and proportions of heterophils increased; haptoglobin concentrations and proportions of lymphocytes, basophils and eosinophils decreased. The variable effects on different immune components demonstrate the complexity of an immune response. We found no evidence that the response differed between annual-cycle stages. The response was independent of baseline measures taken directly upon capture in the field, indicating that birds were facing no immunological ceiling when mounting an immune response. Values of five parameters collected under field conditions were significantly related to values taken under standardised lab conditions. We conclude that multiple parts of the immune system are modulated during an immunological response and that responses are not re-organised throughout the annual cycle.

Key words: inflammation, ecological immunology, acute phase response, lipopolysaccharide (LPS), annual cycle, birds

Introduction

A central premise in ecological immunology is that animals trade off investment into immune function against other competing physiological and behavioral processes (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). However, one important problem that ecoimmunologists face is how to distinguish a reallocation of resources away from the immune system from a reallocation or redistribution within the immune system. Reductions in one or more elements of the immune system do not necessarily equate to a net reduction in immune function because other parts of the immune system might be boosted simultaneously (Adamo, 2004). Simultaneous measurements of multiple immune indices can help address this problem (Adamo, 2004; Matson et al., 2006; Boughton et al., 2011; Buehler et al., 2011; Demas et al., 2011). Yet, understanding trade-offs and interactions within the immune system requires an experimental challenge of the immune system and subsequent quantification of the response using multiple indices (Martin et al., 2006, 2008; Boughton et al., 2011; Pedersen and Babayan, 2011).

The immune system can be experimentally challenged by injection of an endotoxin like lipopolysaccharide (LPS), part of the cell wall of gram-negative bacteria (Owen-Ashley and Wingfield, 2007). As gram-negative bacteria are universal in most environments, an experimental challenge with LPS mimics a functional relevant natural situation. Injection of LPS initiates an immune response by mimicking the first stages of a bacterial infection without actually resulting in sustained disease. This innate response begins minutes after endotoxin detection and defends against threats that breach physical barriers like the skin. Most experimental studies on induced immune responses in free-living birds so far focus on hormonal, behavioural or metabolic changes (Bonneaud et al., 2003; Owen-Ashley and Wingfield, 2006, 2007; Owen-Ashley et al., 2006; Adelman et al., 2010; Hegemann et al., 2012b; reviewed by Hasselquist and Nilsson, 2012). Studies in free-living birds that characterise multiple immunological responses and subsystems simultaneously are lacking so far.

In addition to quantifying which parts of the immune system are affected by a simulated infection, experimental immune challenges can also be used to investigate the consistency of responses through time. Immune responses may be constant among annual-cycle stages, or responses may be seasonally reorganised as a result of trade-offs with other physiological and behavioural demands. Hypotheses relate increased energy demands and decreased resource availability to compromises in costly immune functions and shifts towards less costly immune components (Nelson and Demas, 1996; Nelson, 2004;

Hasselquist, 2007; Martin et al., 2008). Immunological mechanisms aimed at avoiding autoimmunity (Råberg et al., 1998) and preventing oxidative stress (Sorci and Faivre, 2009) might further influence this process. Several studies on non-induced (baseline) immune function indeed show that different indices express different seasonal patterns among and within annual-cycle stages (Nelson and Demas, 1996; Buehler et al., 2008; Pap et al., 2010a, b; Hegemann et al., 2012c). Thus, reorganisation of baseline immune function appears to depend on both environmental conditions and competing biotic processes. Data on seasonal variation in induced immune responses are scarce. However, these are the data needed to verify the hypothesis that free-living birds switch from costly inflammatory responses to highly specific but less costly antibody responses during demanding times (Lee, 2006). A study on captive red knots (*Calidris canutus*) provides evidence for saved costs on inflammatory response during demanding times (Buehler et al., 2009). In contrast, wild skylarks (*Alauda arvensis*) do not modulate the energetic investment in the acute phase response despite seasonal variation in energetic constraints. They maintain similar response throughout the annual cycle as measured by metabolic rate, body temperature, body mass loss, ketone and glucose concentrations (Hegemann et al., 2012b). The detailed knowledge of non-induced (baseline) immune function and energetic costs of an immune challenge in skylarks make this species an ideal candidate for studying the response of multiple immune indices during an immune challenge in different annual-cycle stages. Such a study will also provide a way to test if induced responses are modulated among annual-cycle stages (following patterns of baseline immune function), or if they are maintained throughout the year (reflecting patterns of energetic costs).

Variability in baseline immunological values might also represent important constraints for responses because the ability to mount an immune response might depend on baseline values. For example, baseline haptoglobin concentrations in pigeons (*Columba livia domestica*) have some capacity to predict post-challenge response concentrations (Matson et al., 2012). Great tits (*Parus major*) with high pre-immunisation heterophil/lymphocyte ratios mount weaker antibody responses (Krams et al., 2012). However, it remains to be tested in free-living birds if particularly high (or low) baseline values of a given immune parameter limit the responsiveness of that parameter to an immunological stimulus (i.e. ‘immunological ceiling’). In other words, do individuals with relatively high baseline values respond differently to an immune challenge than birds with relatively low levels? The existence of immunological ceilings can have important implications for the interpretation of values collected from field samples.

In this study, we challenged wild skylarks with LPS and compared them with un-injected controls during five annual-cycle stages to test 1. which immune parameters are affected by an endotoxin challenge, 2. if the immunological response varies among annual-cycle stages, and 3. if baseline values present constraints to the magnitude of the immune response. To capture a broad picture of the immune response we measured different components of immune defence. i) Natural antibodies and complement which agglutinate and lyse foreign cells (Matson et al., 2005) and are measures thought to be unaffected by previous exposure (Ochsenbein and Zinkernagel, 2000). ii) The acute phase protein haptoglobin, which limits the role of plasma iron as nutrient for pathogens and is a initiator of oxidative damage (Murata et al., 2004; Quaye, 2008). iii) The relative abundances of leukocytes, which reflect both innate and acquired components of immune function. Leukocytes are circulating continuously through the blood to maintain a state of readiness and are redistributed in response to immunological stimulation (Feldman et al., 2000). Leukocyte analyses include the heterophil/lymphocyte ratio (hereafter H/L-ratio) which is related to immunological and other stressors (reviewed by Davis et al., 2008). iv) Heat shock proteins (hereafter HSP), which indicate stress (Martinez-Padilla et al., 2004) and have been suggested to be a potential indicator for autoimmune risk (Hasselquist and Nilsson, 2012). Furthermore they play an important role in modulating innate and acquired immunity (Pockley, 2003; Pockley et al., 2008) through their capacity to activate complement and trigger the release of inflammatory cytokines (Calderwood et al., 2007).

Methods

Study subjects

We caught adult skylarks during five annual-cycle stages in the northern Netherlands in 2008 focusing on our study population at the Aekingerzand (N 52°55’; E 6°18’; (Hegemann et al., 2012b). Some skylarks in our study population migrate; others winter locally and are accompanied by birds that breed further north and east (Hegemann et al., 2010). We caught birds during breeding in June and July (9 males, 6 females, hereafter m & f), molt in August and September (12 m, 7 f), autumn migration in October (12 m, 12 f), winter in December and January (14 m, 3 f), and spring migration in March (17 m, 9 f). Birds were sexed biometrically, and in some doubtful cases molecularly (Hegemann et al., 2012a). For details on catching see Hegemann et al. (2012b). All individuals were fully grown. Because skylarks undergo a complete post-nuptial moult in August-September, age classes could not be distinguished. Since skylarks breed in their first year (Hegemann unpublished data) and both

young and adult birds are known to migrate (van Dobben and Mörzer Bruyna, 1939; Hegemann et al., 2010), we have no indications that an age bias between stages exists and could influence the interpretation of the results.

Sampling protocol

When catching skylarks in the field we collected blood (~150 uL) into heparinised capillary tubes from the brachial vein after capture (median: 5 min; range: 2.25-30 min) to minimize any impacts of handling stress (Buehler et al., 2008). We then took structural measurements. We refer to measurements from these samples as “field values”.

After capture, birds were brought into captivity (cages 30x40x60 cm). During the breeding season, when skylarks are territorial, birds were housed individually. During the non-breeding seasons, when skylarks live in socially interacting flocks, birds were housed in small groups (≤ 3 birds per cage). Even though the captivity period was short, we attempted to avoid a potential seasonal bias by using conditions that reflected current social conditions in the wild. Birds had access to *ad libitum* water and food (mealworms and seeds) until 4:30 p.m. on the experimental-protocol start day (for details, see Hegemann et al., 2012b).

We started the experimental protocol with isolating birds in a dark box for 1 h without food and water. At 5:30 p.m. we injected experimental birds with 2.5 mg LPS in 10 ml PBS per kg body mass in their abdominal cavities (Hegemann et al., 2012b). Control birds remained un-injected, because puncturing the skin and underlying tissues for injecting only a vehicle (i.e. PBS) can result in inflammation (K. Klasing and B. Helm, personal communications). Consequently, the experimental responses must be viewed as a result of both LPS and injection procedure. This combination does not pose interpretational problems for our study since our central interest is immune response and not the effects of LPS per se. After injection the experimental birds and their corresponding controls were put into dark boxes (metabolic chambers) where they spent the night at thermo neutral conditions (Hegemann et al., 2012b). The next morning at 6:30 a.m. (13 h after injecting experimental birds) we collected another blood sample (150 uL) within 10 min of removing birds from boxes. The 13 hour interval between start of the experiment and taking a blood sample was based on the ability to match metabolic measurements with blood sampling in a time frame in which most physiological and behavioural reactions occur (e.g. Owen-Ashley et al., 2006; Adelman et al., 2010; Burness et al., 2010) and with the need to return birds, especially during the breeding season, quickly to the field.

From each blood sample (field and lab), we used a small drop to make blood smears for leukocyte enumeration. The remainder of each sample was centrifuged at 7000 rpm for 10 min. Plasma and red blood cells were separated and stored at -20°C. Upon completion of the protocol, birds were released at the site of capture.

Because the stress of short-term captivity might affect immune function differently in different seasons (Sapolsky et al., 2000; Martin, 2009), we evaluated the effects of stress throughout the annual cycle. For this purpose, we used H/L-ratios (Gross and Siegel, 1983; Vleck et al., 2000; Davis, 2005; Huff et al., 2007) and HSP70 concentrations (Martinez-Padilla et al., 2004; Bourgeon et al., 2006). We favored these two independent and functionally integrative methods over concentrations of specific hormones (e.g., corticosterone) since the effects of hormones can depend strongly on levels of binding globulins and other related factors (Deviche et al., 2001; Lynn et al., 2003). In order to separate the stress of captivity and general protocols (experienced by all birds) from the stress response of the immune challenge (experienced only by experimental birds), we explored seasonal variation in stress response in the control birds. With these birds, we calculated differences between values from field samples and morning lab samples (Δ H/L-ratio and Δ HSP70). While, both indices increased as expected due to the stress associated with captivity, we found no significant seasonal pattern in this captivity-related stress response (Δ H/L-ratio $\chi^2_{4,46}$=4.04, p=0.40; Δ HSP70 $\chi^2_{4,45}$=0.40, p=0.53). Furthermore, there were no differences in the metabolic effects (O2 consumption and nightly mass loss) of an LPS-injection when comparing birds that were held in captivity for a few hours according to the protocol used in this study and birds acclimated to captivity for 55 days (Hegemann et al. 2012b). Thus we have no evidence that the immune response we experimentally triggered was masked by any stress responses resulting from the short time in captivity. Experiments were performed under license DEC5219B of the Institutional Animal Care and Use Committee of the University of Groningen.

Immune assays

We used a hemolysis-hemagglutination assay (rabbit red blood cells, B-0009H; Harlan, Leicestershire, United Kingdom) to quantify titers of complement-like lytic enzymes (i.e., lysis) and non-specific natural antibodies (i.e., agglutination) in plasma (Matson et al., 2005; Hegemann et al., 2012c). Scans of individual samples were randomized among all plates and scored blindly to treatment and season (by AH). A plasma standard was run in duplicate in all plates. On average, variation (standard deviation) within (0.4 lysis titers and 0.7 agglutination

titers) and among (0.5 lysis titers and 1.1 agglutination titers) plates is similar to that originally described by Matson et al. (2005). We used a commercially available colorimetric assay kit (TP801; Tri-Delta Diagnostics, NJ, USA) to quantify haptoglobin concentrations (mg ml^{-1}) in plasma samples (Hegemann et al., 2012c; Matson et al., 2012). Blood smears were examined by one person (C. Gottland), who was blind to treatment and season. The first 100 white blood cells (WBC) per slide were identified and counted as lymphocytes, heterophils, basophils, monocytes or eosinophils (Hegemann et al., 2012c).

Heat shock proteins (Hsp70)

Cell lysates were obtained as in Tomas et al. (2004) and total protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as the standard. Concentrations of Hsp70 were determined from the cell lysates by means of an enzyme linked immunosorbent assay (ELISA) using the protocol described by Mahmoud and Edens (, 2003). Briefly, 100 ul of samples (dilution 1:10), standards (0-50 ng recombinant human Hsp70) and a positive control (HeLa Cell Lysate) were coated in duplicate in 96-well immunoplates at 4ºC overnight. After blocking non-specific binding sites, plates were incubated 1 h with100 ul of anti-Hsp70 monoclonal antibody (H5147; Sigma) diluted 1:1000. Following washing, plates were incubated with 100 µl of 1:5000 alkaline phosphatase conjugated goat anti-mouse IgG polyclonal antibody (SAB-101; Stressgen) for 1h. Finally we added 1 mg ml^{-1} pNPP in coating buffer for 30 min, and read the absorbance of individual wells at 405 nm with a microplate reader (PowerWave; BioTek). Hsp70 concentration was calculated from the standard curve. All final Hsp70 values were standardized by dividing Hsp70 concentration by total protein and normalized according to plate-specific positive controls to facilitate inter-plate comparisons. Based on samples run in duplicate, the mean intra-assay coefficient of variation was 5.9% and mean inter-assay coefficient of variation was 6.6%.

Statistics

We compared experimental and control groups for each response variable using linear models analysed in R, version 2.9.2 (R Development Core Team, 2009). We included treatment, annual-cycle stage, sex and all possible interactions as explanatory variables. White blood cell types were analysed with generalized linear models with a quasi-binomial approach and F-tests. These tests incorporated the counts of one cell type and the total remaining WBC

number (e.g. basophils against the sum of heterophils, lymphocytes, monocytes and eosinophils using the 'c-bind' function in R). H/L-ratios were tested in a linear model.

To test if baseline values as taken upon capture in the field affected the outcome of the experiment, we calculated the individual deviation from season- and sex-specific means. We included this sex- and season-independent term and the interaction with treatment in all analyses. A ceiling in the ability to respond would be indicated by a significant interaction. A significant main effect would indicate that individuals express consistent immune parameters in the field and in the lab after having gone through a standardised experimental protocol in the preceding 14 hour period.

We always started with the full model and simplified it using backwards elimination based on log likelihood ratio test with $P < 0.05$ as selection criterion ("drop1" in R) until reaching the minimal adequate model. Model assumptions were checked using the residuals of the final model. Sample sizes, which are provided in the figures, differ among response variables due to insufficient plasma volume. Graphs were made using the package "gplots" (Warnes, 2009). Baseline values, measured in the field just after capture, did not differ significantly between experimental and control groups in any of the 10 parameters measures (always $p > 0.25$).

Results

Immunological responses after endotoxin challenge

Compared with control birds, injected skylarks exhibited significantly increased lysis 13 hours after an endotoxin challenge (Fig. 1A), but experimental and control birds did not differ significantly in terms of agglutination (Fig. 1B). Concentrations of haptoglobin were significantly lower in endotoxin-challenged birds (Fig. 1C). Experimental birds had significantly higher proportions of heterophils than control birds (Fig. 1E). The proportions of lymphocytes, basophils and eosinophils were lower in experimental birds (Fig. 1F,B,I). The H/L-ratio, the proportion of monocytes and concentrations of Hsp70 were not affected by the endotoxin challenge (Fig. 1D,H,J). Thus, experimental birds differed significantly from control birds in 6 of the 10 physiological parameters (Table 1). We never found a significant difference between the sexes in their response to the endotoxin challenge (interaction treatment*sex always $\chi^2/F < 1.27$, $p > 0.26$). Independent of treatment, males and females differed significantly in 2 of the 10 parameters in the morning after the injection (Table 1). Females exhibited significantly higher proportions of eosinophils among their WBCs

(females 10.6%, males 5.9%). Males had significantly higher haptoglobin concentrations (15.9%) and statistically-marginally higher lysis titers (16.1%) than females.

Seasonal variation in immune response

Based on samples taken in the morning in the lab, Skylarks showed significant differences among annual-cycle stages in 8 of the 10 measured parameters (Table 1, Fig. 1A-J), but the immune response after the endotoxin challenge did not differ among annual-cycle stages: the interaction between annual-cycle stage and endotoxin challenge was not significant for any of the measured parameters (Table 1, Fig. 1A-J).

Immunological ceiling and individual consistency

The response to the endotoxin challenge was independent of field immune values. Changes in immune parameters after the endotoxin challenge were always independent of the corresponding value measured in the field (interaction treatment*deviation of the field value always $\chi^2/F < 3.12$, $p > 0.08$).

After accounting for treatment, individual skylarks showed values that were consistent between deviation of the field values and morning samples for 5 parameters. With lysis titer, haptoglobin concentration, the proportion of heterophils, lymphocytes and eosinophils, we found significant positive relationships between the field values (corrected for sex and season-variation) and the morning values (Table 1). There was no significant relationship between deviation of the field values and morning values for agglutination titer, the Hsp70 concentration, the H/L-ratio and the proportion of basophils and monocytes (Table 1).

Discussion

Skylarks exhibited complex and multifaceted responses when experimentally challenged with endotoxin. Thirteen hours post-injection, some parameters increased (lysis titer, heterophil proportion), others decreased (haptoglobin concentration, proportion of lymphocytes, basophils and eosniophils) and others were unchanged (agglutination titers, H/L-ratio, Hsp70 concentration and proportion of monocytes). The complexity of the immune response to endotoxin highlights methodological complications for ecoimmunologists trying to interpret samples and data collected from birds in the field. For example, relatively high or low values of one immune parameter should be interpreted cautiously when measured in isolation of other parameters. Despite its complexity, we found no evidence for seasonal reorganization

of the immune response, which was consistent among five annual-cycle stages. Furthermore, we found no evidence for an immunological ceiling; birds showed similar responses to an immunological challenge regardless of their baseline values measured from values collected in the field. After accounting for treatment, individuals showed consistent values in samples from the field and samples from the lab with lysis titer, haptoglobin concentration, H/L-ratio, and eosinophil proportions. This suggest that these measures are relatively robust against possible sources of variation like time of day, activity and nutritional status.

Physiological responses after immune challenge

One particularly surprising result that highlights the complications associated with assigning a single immune parameter, relates to haptoglobin. LPS-injected skylarks exhibited 13 hours post-challenge significantly lower concentrations of haptoglobin compared to un-injected control birds. Haptoglobin is an acute phase protein that is released from the liver during a pathogenic challenge. Normally in birds, concentrations of haptoglobin or iron-binding functional equivalents increase in association with inflammation (Thomas, 2000; van de Crommenacker et al., 2010). Our finding suggests that in skylarks haptoglobin might be more appropriately classified as a negative, rather than a positive, acute phase protein when measured 13 hours after the endotoxin challenge. Any functional relevance of the observed reductions in concentrations of this protein, which sequesters iron, remains to be elucidated. Notably, compared to other species that have been similarly assayed, skylarks maintain relatively high circulating concentrations of baseline haptoglobin (Matson, 2006). The decrease we observed in skylarks following LPS injection may relate to dissimilar rates of haptoglobin production and consumption in this species. This result also suggests a greater reliance of skylarks on constitutive (rather than induced) production of this bacteriostatic and antioxidant molecule. Testing these possibilities will require more detailed studies (e.g. with more frequent sample time points) of the LPS-induced inflammation time-course in skylarks and other species of birds. However, results of a pilot study showed that haptoglobin concentrations in skylarks decreased by 13 hours and remained low at 24 hours after an LPS injection (unpublished data of KDM).

Lysis titers of skylarks increased following endotoxin challenge, but there was no difference in agglutination (natural antibody) titers between control and experimental birds. Antibody production normally requires days not hours. Thus it is not surprising that agglutination titres did not differ between groups 13 hours post challenge. The increase in lysis titers during infection points to another important topic of ecoimmunology: High values

are not necessarily better (De Coster et al., 2011). Instead, values should be viewed in relation to the immunological status, e.g. by measuring parasite infection rates (Pap et al., 2011).

Circulating leukocytes are important for the protection against invading microorganisms. During immune responses redistributions of leukocytes populations occur (Gehad et al., 2002). In skylarks, proportions of heterophils increased and proportions of lymphocytes decreased following endotoxin challenge. Since heterophils relate to innate immunity and lymphocytes relate to acquired immunity, the innate inflammatory response we elicited could primarily affect heterophil concentrations (De Boever et al., 2009). However, upon an immune challenge a redistribution of peripheral blood lymphocytes to secondary lymphoid organs occurs (Gehad et al., 2002), and this process could contribute to reduced numbers of lymphocytes in the circulating blood. Basophils are one of the first leukocyte types to enter tissue during an early inflammatory response in birds (Katiyar et al., 1992). The decreased proportion of basophils suggests that these cells are no longer circulating in the peripheral blood and have migrated into the tissue at the LPS injection site. Given this biological functioning, we are confident that all observed changes in leukocyte profiles are meaningful, despite two relatively high p-values, which should be viewed with added caution in multiple testing (Moran, 2003).

We found no difference in concentrations of intracellular Hsp70 concentrations between control and experimental skylarks. Autoimmune reactions caused by physiological stress during an immune response might be an important cost of immunity (Råberg et al., 1998) and heat shock protein quantification could be an indirect way to assess the potential risks of autoimmune reactions (Hasselquist and Nilsson, 2012). Our data do not provide any evidence for increased physiological stress during the immune response to an endotoxin. Heat shock proteins also have more direct immunological functions. Extracellular levels of certain types, such as Hsp60 and Hsp70, exhibit modulating effects on innate and acquired immunity (Pockley, 2003; Pockley et al., 2008) and these proteins are involved in the activation of complement and release of cytokines (Calderwood et al., 2007). In rats intra- and extracellular heat shock protein concentrations are correlated (Fleshner et al., 2004). The lack of change in intracellular heat shock protein concentrations in immunologically challenged skylarks, despite increased complement activity, might indicate different relationships in wild birds. Detailed studies of both extracellular and intracellular heat shock protein concentrations at multiple time points following an immunological challenge are required to reveal the causes and consequences of heat shock protein variation in wild birds.

Consistent responses throughout the annual cycle

We found no evidence that the response of the immune system to endotoxin differed among 5 annual-cycle stages experienced by skylarks. The reaction to the endotoxin challenge also did not differ between the sexes. These findings are in line with our previous finding that energetic components of an acute phase response (as measured by metabolic rate, body temperature, body mass loss, ketone and glucose concentrations) are not seasonally modulated in this species (Hegemann et al., 2012b). After statistical correction of the treatment effects, it is noteworthy that we found seasonal differences in eight of ten immunological parameters that we measured using samples collected in the lab. These data support our earlier findings that free-living skylarks modulate their baseline immune function among annual-cycle stages as measured on samples collected upon capture in the field (Hegemann et al., 2012c). Taken together, our results suggest that skylarks do modulate baseline values of immune function, as has been described for other species (Buehler et al., 2008; Pap et al., 2010a, b). However, both the energetic (Hegemann et al., 2012b) and the immunological (this study) consequences of an endotoxin challenge are constant throughout the year, independent of other annual-cycle demands and equal for both sexes. This suggests that mounting this type of immune response is crucial to survival and cannot be compromised. Only baseline defences can be traded off with other demands. This conclusion further highlights the interpretational limitations and the importance of distinguishing between baseline values and induced responses when studying ecological immunology (Adamo, 2004; Hegemann et al., 2012b, c).

This finding - that responses to an LPS-injection were constant throughout the annual cycle - necessitates a short discussion of two methodological points. First, skylarks in our study population are partial migrants: some birds migrate, others winter locally and get accompanied by birds from more northern and eastern breeding populations (Hegemann et al., 2010). With our year-round study focused on the breeding location, we potentially caught a mixture of birds from different populations during winter and migration. However, similar coefficients of variation (CV) throughout the annual cycle for each of the response variables demonstrate that any (unmeasured) variability in the composition of the sampled populations did not translate to differences in immunological variability. It is also supported by our data on baseline immune function (Hegemann et al, 2012c) and energetic effects of an immune challenge (Hegemann et al, 2012b). Consequently, immune responses to LPS-injection by skylarks seem to be relatively independent of breeding location and more dependent on

current local conditions. Second, as with any decision based on statistics, our acceptance of our null hypothesis was influenced by sample size, variance and effect size. Small sizes can undermine some hypotheses via type II errors or false negatives, but this is unlikely to occur consistently (e.g., as with all 10 dependent variables). Large sample sizes can minimize this type of error but sometimes detect differences that lack biological relevance. In principle, power analyses could provide insight into these issues, but in practice such analyses require precise input regarding strength, direction, timing, and variance, none of which are available for the interaction between LPS-treatment and annual-cycle stage. In light of these points, we feel confident that our sample sizes are sufficient to accept our null hypothesis and draw conclusions accordingly.

Immunological ceiling and individual consistency

The strength of the immunological response as measured by 10 parameters was independent of the corresponding values measured upon capture in the field. Thus birds did not face an immunological ceiling, and similar immune responses were mounted regardless of an individual's baseline values. A corresponding pattern also exists at the population level as the immune response was independent of seasonal patterns of baseline values (see above).

Discarding the effect of the endotoxin challenge, several immune indices (lysis titer, haptoglobin concentration, the proportion of heterophils, lymphocytes and eosinophils) show a significant correlation between values from samples collected in the field and values from samples collected in the lab after birds had gone through a standard 14 hour protocol. After correction for treatment these parameters showed a positive correlation between the field and morning values. These results indicate that individuals exhibit consistent values in the face of variable environmental and physiological conditions. Birds sampled in the morning in the lab exhibited highly standardized conditions (temperature and light regime, food and water availability). However, in the field, at least some conditions varied, like time of the day and previous activity. While many factors are known to affect immune indices (e.g. diurnal patterns (Navarro et al., 2003; Martinez-Padilla, 2006); flight behavior (Matson et al., 2012)), skylarks showed consistent values for these five parameters independent of the conditions under which they were taken. This suggests that these indices are robust against short term (hours) biotic and abiotic environmental variation. Consequently they are suitable for ecoimmunologists interested in longer term environmental variation or in the immunological status of their study subjects.

Acknowledgements

Many volunteers helped catch skylarks at various times of the year. We are thankful to everyone, especially Kees van Eerde, Rob Voesten, Martin Keiser, Richard Ubels, and Chris Trierweiler. Cecile Gottland and Emmanuelle Gilot performed the slide counts. Isabel Piedad provided assistance in Hsp analysis. Ido Pen gave valuable advice on statistical tests. Discussions with Christiaan Both and his comments on early drafts were of great help. Debbie M. Buehler, Lusia Mendes and three anonymous reviewers commented on an earlier version of the manuscript.

Funding

This work was supported by a grant from BirdLife Netherlands to BIT, by grants from the Schure-Beijerinck-Popping Fonds to AH, by a grant from the Regional Government of Extremadura [POS700013] and from University of Extremadura [A7-22/09] to AV. BIT was supported by a Rosalind Franklin Fellowship and the Netherlands Organization for Scientific Research. KDM was also supported by a Veni fellowship [863.08.026].

Figure 1: Effects of an endotoxin challenge on 10 immune parameters in skylarks as measured from the blood after 13 h after the experimental start. Experimental birds were injected with LPS; control birds were un-injected. Means and standard errors are shown; numbers in bars represent sample sizes. There was never a significant treatment*season interaction (all $p > 0.08$). LPS injection had a significant effect on lysis titers, haptoglobin concentrations and the proportion of lymphocytes, basophils and eosinophils. Full statistical details can be found in table 1.

References

Adamo, S. A. (2004). How should behavioural ecologists interpret measurements of immunity? *Anim. Behav.* **68,** 1443-1449.

Adelman, J. S., Cordoba-Cordoba, S., Spoelstra, K., Wikelski, M. and Hau, M. (2010). Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. *Funct. Ecol.* **24,** 813-823.

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B. and Sorci, G. (2003). Assessing the Cost of Mounting an Immune Response. *Am. Nat.* **161,** 367-379.

Boughton, R. K., Joop, G. and Armitage, S. A. O. (2011). Outdoor immunology: methodological considerations for ecologists. *Funct. Ecol.* **25,** 81-100.

Bourgeon, S., Martinez, J., Criscuolo, F., Le Maho, Y. and Raclot, T. (2006). Fasting-induced changes of immunological and stress indicators in breeding female eiders. *Gen. Comp. Endocrinol.* **147,** 336-342.

Buehler, D. M., Bhola, N., Barjaktarov, D., Goymann, W., Schwabl, I., Tieleman, B. I. and Piersma, T. (2008). Constitutive immune function responds more slowly to handling stress than corticosterone in a shorebird. *Physiol. Biochem. Zool.* **81,** 673-681.

Buehler, D. M., Encinas-Viso, F., Petit, M., Vezina, F., Tieleman, B. I. and Piersma, T. (2009). Limited access to food and physiological trade-offs in a long-distance migrant shorebird. II. Constitutive immune function and the acute-phase response. *Physiol. Biochem. Zool.* **82,** 561-571.

Buehler, D. M., Piersma, T., Matson, K. and Tieleman, B. I. (2008). Seasonal redistribution of immune function in a migrant shorebird: annual-cycle effects override adjustments to thermal regime. *Am. Nat.* **172,** 783-796.

Buehler, D. M., Versteegh, M. A., Matson, K. D. and Tieleman, I. (2011). One Problem, Many Solutions: Simple Statistical Approaches Help Unravel the Complexity of the Immune System in an Ecological Context. *Plos One* **6,** e18592.

Burness, G., Armstrong, C., Fee, T. and Tilman-Schindel, E. (2010). Is there an energetic-based trade-off between thermoregulation and the acute phase response in zebra finches? *J. Exp. Biol.* **213,** 1386-1394.

Calderwood, S. K., Mambula, S. S., Gray, P. J., Jr. and Therlault, J. R. (2007). Extracellular heat shock proteins in cell signaling. *FEBS Lett.* **581,** 3689-3694.

Davis, A. K. (2005). Effect of handling time and repeated sampling on avian white blood cell counts. *J. Field Ornithol.* **76,** 334-338.

Davis, A. K., Maney, D. L. and Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct. Ecol.* **22,** 760-772.

De Boever, S., Croubels, S., Meyer, E., Sys, S., Beyaert, R., Ducatelle, R. and De Backer, P. (2009). Characterization of an intravenous lipopolysaccharide inflammation model in broiler chickens. *Avian Pathol.* **38,** 403-411.

De Coster, G., Verhulst, S., Koetsier, E., De Neve, L., Briga, M. and Lens, L. (2011). Effects of early developmental conditions on innate immunity are only evident under favourable adult conditions in zebra finches. *Naturwissenschaften* **98,** 1049-1056.

Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. and French, S. S. (2011). Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts. *J. Anim. Ecol.* **80,** 710-730.

Deviche, P., Breuner, C. and Orchinik, M. (2001). Testosterone, corticosterone, and photoperiod interact to regulate plasma levels of binding globulin and free steroid hormone in Dark-eyed Juncos, *Junco hyemalis. Gen. Comp. Endocrinol.* **122,** 67-77.

Feldman, B. F., Zinkl, J. G. and Jain, N. C. (2000). *Schalm's Veterinary Hematology*, pp. 1344. Philadelphia, London: Lippincott Williams & Wilkins.

Fleshner, M., Campisi, J., Amiri, L. and Diamond, D. (2004). Cat exposure induces both intra- and extracellular Hsp72: the role of adrenal hormones. *Psychoneuroendocrinology* **29,** 1142-1152.

Gehad, A. E., Lillehøj, H. S., Hendricks, G. L. and Mashaly, M. M. (2002). Initiation of humoral immunity. II. The effects of T-independent and T-dependent antigens on the distribution of lymphocyte populations. *Dev. Comp. Immunol.* **26,** 761-771.

Gross, W. B. and Siegel, H. S. (1983). Evaluation of the Heterophil Lymphocyte Ratio as a Measure of Stress in Chickens. *Avian Diseases* **27,** 972-979.

Hasselquist, D. (2007). Comparative immunoecology in birds: hypotheses and tests. *J. Ornithol.* **148,** S571-S582.

Hasselquist, D. and Nilsson, J. A. (2012). Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? *Anim. Behav.* **83,** 1303-1312.

Hegemann, A., van der Jeugd, H. P., de Graaf, M., Oostebrink, L. L. and Tieleman, B. I. (2010). Are Dutch Skylarks partial migrants? Ring recovery data and radio-telemetry suggest local coexistence of contrasting migration strategies. *Ardea* **98,** 135-143.

Hegemann, A., Voesten, R., van Eerde, K., van der Velde, M. and Tieleman, B. I. (2012a). The use of tongue spots for aging and wing length for sexing Skylarks *Alauda arvensis* - A critical evaluation. *Ring. Migrat.* **27,** 7-12.

Hegemann, A., Matson, K. D., Versteegh, M. A. and Tieleman, B. I. (2012b). Wild skylarks seasonally modulate energy budgets but maintain energetically costly inflammatory immune responses throughout the annual cycle. *PLoS ONE* **7,** e36358.

Hegemann, A., Matson, K. D., Both, C. and Tieleman, B. I. (2012c). Immune function in a free-living bird varies over the annual cycle, but seasonal patterns differ between years. *Oecologia* **170,** 605-618.

Huff, G., Huff, W., Rath, N., Donoghue, A., Anthony, N. and Nestor, K. (2007). Differential effects of sex and genetics on behavior and stress response of turkeys. *Poultry Sci.* **86,** 1294-1303.

Katiyar, A. K., Vegad, J. L. and Awadhiya, R. P. (1992). Pathology of inflammatory-reparative response in punched wounds of the chicken skin. *Avian Pathol.* **21,** 471-480.

Krams, I., Vrublevska, J., Cirule, D., Kivleniece, I., Krama, T., Rantala, M. J., Sild, E. and Hõrak, P. (2012). Heterophil/lymphocyte ratios predict the magnitude of humoral immune response to a novel antigen in great tits (*Parus major*). *Comp. Biochem. Phys. A* **161,** 422-428.

Lee, K. A. (2006). Linking immune defenses and life history at the levels of the individual and the species. *Integr. Comp. Biol.* **46,** 1000-1015.

Lochmiller, R. L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88,** 87-98.

Lynn, S. E., Breuner, C. W. and Wingfield, J. C. (2003). Short-term fasting affects locomotor activity, corticosterone, and corticosterone binding globulin in a migratory songbird. *Horm. Behav.* **43,** 150-157.

Mahmoud, K. Z. and Edens, F. W. (2003). Influence of selenium sources on age-related and mild heat stress-related changes of blood and liver glutathione redox cycle in broiler chickens (*Gallus domesticus*). *Comp. Biochem. Phys. B* **136,** 921-934.

Martin, L. B. (2009). Stress and immunity in wild vertebrates: timing is everything. *Gen. Comp. Endocrinol.* **163,** 70-76.

Martin, L. B., Weil, Z. M. and Nelson, R. J. (2006). Refining approaches and diversifying directions in ecoimmunology. *Integr. Comp. Biol.* **46,** 1030-1039.

Martin, L. B., Weil, Z. M. and Nelson, R. J. (2008). Seasonal changes in vertebrate immune activity: mediation by physiological trade-offs. *Philos. Trans. R. Soc. Lond. B* **363,** 321-339.

Martinez-Padilla, J. (2006). Daytime variation in T-cell-mediated immunity of Eurasian kestrel *Falco tinnunculus* nestlings. *J. Avian Biol.* **37,** 419-424.

Martinez-Padilla, J., Martinez, J., Davila, J. A., Merino, S., Moreno, J. and Millan, J. (2004). Within-brood size differences, sex and parasites determine blood stress protein levels in Eurasian Kestrel nestlings. *Funct Ecol* **18,** 426-434.

Matson, K. D., Horrocks, N. P. C., Versteegh, M. A. and Tieleman, B. I. (2012). Baseline haptoglobin concentrations are repeatable and predictive of certain aspects of a subsequent experimentally-induced inflammatory response. *Comp. Biochem. Phys. A* **162,** 7-15.

Matson, K. D., Ricklefs, R. E. and Klasing, K. C. (2005). A hemolysis-hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev. Comp. Immunol.* **29,** 275-286.

Matson, K. (2006). Are there differences in immune function between continental and insular birds? *Proc. R. Soc. B* **273,** 2267-2274.

Matson, K., Cohen, A., Klasing, K., Ricklefs, R. and Scheuerlein, A. (2006). No simple answers for ecological immunology: relationships among immune indices at the individual level break down at the species level in waterfowl. *Proc. R. Soc. B* **273,** 815-822.

Matson, K. D., Horrocks, N. P. C., Tieleman, B. I. and Haase, E. (2012). Intense flight and endotoxin injection elicit similar effects on leukocyte distributions but dissimilar effects on plasma-based immunological indices in pigeons. *J. Exp. Biol.* **215:** 3734-3741.

Moran, M. D. (2003). Arguments for rejecting the sequential Bonferroni in ecological studies. *Oikos* **100,** 403-405.

Murata, H., Shimada, N. and Yoshioka, M. (2004). Current research on acute phase proteins in veterinary diagnosis: an overview. *Vet. J.* **168,** 28-40.

Navarro, C., Marzal, A., de Lope, F. and Møller, A. P. (2003). Dynamics of an immune response in house sparrows *Passer domesticus* in relation to time of day, body condition and blood parasite infection. *Oikos* **101,** 291-298.

Nelson, R. J. (2004). Seasonal immune function and sickness responses. *Trends in Immunology* **25,** 187-192.

Nelson, R. J. and Demas, G. E. (1996). Seasonal Changes in Immune Function. *Q. Rev. Biol.* **71,** 511-548.

Norris, K. and Evans, M. R. (2000). Ecological immunology: life history trade-offs and immune defense in birds. *Behav. Ecol.* **11,** 19-26.

Ochsenbein, A. F. and Zinkernagel, R. M. (2000). Natural antibodies and complement link innate and acquired immunity. *Immunology Today* **21,** 624-630.

Owen-Ashley, N. T., Turner, M., Hahn, T. P. and Wingfield, J. C. (2006). Hormonal, behavioral, and thermoregulatory responses to bacterial lipopolysaccharide in captive and free-living white-crowned sparrows (*Zonotrichia leucophrys gambelii*). *Horm. Behav.* **49,** 15-29.

Owen-Ashley, N. T. and Wingfield, J. C. (2007). Acute phase responses in passerine birds: characterization and seasonal variation. *J. Ornithol.* **148,** 583-591.

Owen-Ashley, N. T. and Wingfield, J. C. (2006). Seasonal modulation of sickness behavior in free-living northwestern song sparrows (*Melospiza melodia morphna*). *J. Exp. Biol.* **209,** 3062-3070.

Pap, P. L., Czirjak, G. A., Vagasi, C. I., Barta, Z. and Hasselquist, D. (2010a). Sexual dimorphism in immune function changes during the annual cycle in house sparrows. *Naturwissenschaften* **97,** 891-901.

Pap, P. L., Vagasi, C. I., Tokolyi, J., Czirjak, G. A. and Barta, Z. (2010b). Variation in haematological indices and immune function during the annual cycle in the Great Tit *Parus major*. *Ardea* **98,** 105-112.

Pap, P. L., Vagasi, C. I., Czirjak, G. A., Titilincu, A., Pintea, A., Osvath, G., Fueloep, A. and Barta, Z. (2011). The Effect of Coccidians on the Condition and Immune Profile of Molting House Sparrows (*Passer Domesticus*). *Auk* **128,** 330-339.

Pedersen, A. B. and Babayan, S. A. (2011). Wild immunology. *Mol. Ecol.* **20,** 872-880.

Pockley, A. G. (2003). Heat shock proteins as regulators of the immune response. *Lancet* **362,** 469-476.

Pockley, A. G., Muthanal, M. and Calderwood, S. K. (2008). The dual immunoregulatory roles of stress proteins. *Trends Biochem. Sci.* **33,** 71-79.

Quaye, I. K. (2008). Haptoglobin, inflammation and disease. *Trans. R. Soc. Trop. Med. Hyg.* **102,** 735-742.

R Development Core Team. (2009). *R: A Language and Environment for Statistical Computing*. Vienna, Austria http://www.R-project.org: R Foundation for Statistical Computing.

Råberg, L., Grahn, M., Hasselquist, D. and Svensson, E. (1998). On the adaptive significance of stress-induced immunosuppression. *Proc. R. Soc. B* **265,** 1637-1641.

Sapolsky, R. M., Romero, L. M. and Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* **21,** 55-89.

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11,** 317-321.

Sorci, G. and Faivre, B. (2009). Inflammation and oxidative stress in vertebrate host-parasite systems. *Philos. Trans. R. Soc. Lond. B* **364,** 71-83.

Thomas, J. S. (2000). Overview of Plasma Proteins. In (ed. B. F. Feldman, J. G. Zinkl, N. C. Jain and O. W. Schalm), pp. 891-1344. Philadelphia; London: Lippincott Williams & Wilkins.

Tomás, G., Martinez, J. and Merino, S. (2004). Collection and analysis of blood samples to detect stress proteins in wild birds. *J. Field Ornithol.* **75,** 281-287.

van de Crommenacker, J., Horrocks, N. P. C., Versteegh, M. A., Tieleman, B. I., Komdeur, J. and Matson, K. D. (2010). Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists. *J. Exp. Biol.* **213,** 3527-3535.

van Dobben, W. H. and Mörzer Bruyna, M. F. (1939). Zug nach Alter und Geschlecht an niederländischen Leuchttürmen. *Ardea* **28,** 61-71.

Vleck, C. M., Vertalino, N., Vleck, D. and Bucher, T. L. (2000). Stress, corticosterone, and heterophil to lymphocyte ratios in free-living Adelie Penguins. *Condor* **102,** 392-400. **Warnes, G. R.** (2009). *Various R Programming Tools for Plotting Data*: R package version 2.7.4.

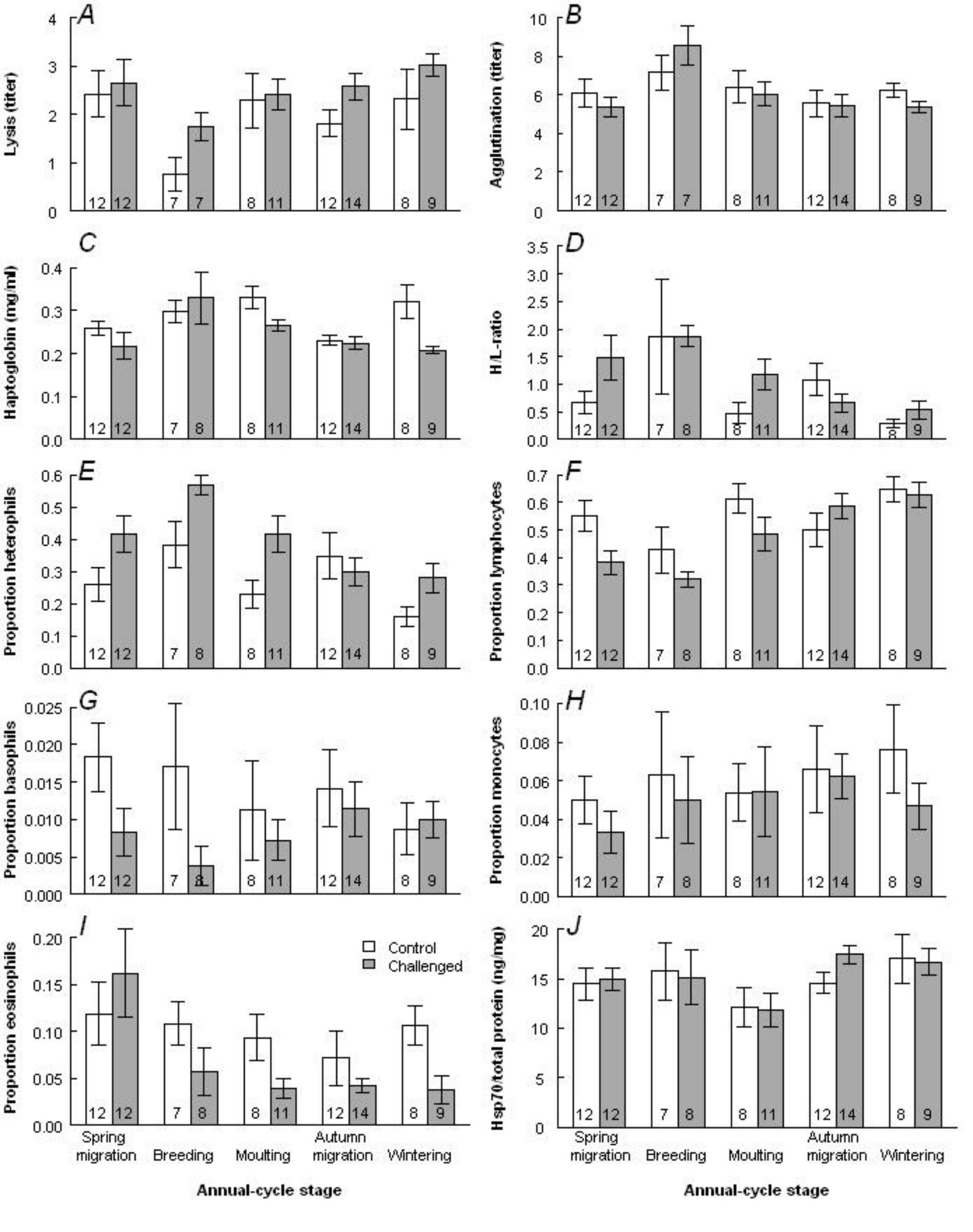
A
Lysis (titer)
B
Agglutination (titer)
C
Haptoglobin (mg/ml)
D
H/L-ratio
E
Proportion heterophils
F
Proportion lymphocytes
G
Proportion basophils
H
Proportion monocytes
I
Proportion eosinophils
J
Hsp70/total protein (ng/mg)
Control
Challenged
Spring migration
Breeding
Moulting
Autumn migration
Wintering
Annual-cycle stage

Table 1: Statistics and coefficients of linear models for 10 measured parameters in skylarks. Experimental birds were injected with LPS; control birds were un-injected. Results are from linear models after removing all non-significant terms (P>0.05).

Trait	Treatment				Season			Sex				Field value deviation[§]				Treatment x season		
	df	Chi²/F	p	β[‡]	df	Chi²/F	p	df	Chi²/F	p	β[†]	df	Chi²/F	p	β	df	Chi²/F	p
Lysis titer	98,1	8.35	**0.004**	0.679	98,4	13.68	**0.008**	98,1	3.83	0.050	-0.481	98,1	9.14	**0.003**	0.365	98,1	4.39	0.356
Agglutination titer	99,1	0.38	0.535		99,4	13.33	**0.010**	99,1	2.16	0.142		99,1	0.42	0.516		99,1	3.53	0.474
Haptoglobin	100,1	12.36	<**0.001**	-0.172	100,4	22.99	<**0.001**	100,1	7.54	**0.006**	-0.140	100,1	8.87	**0.003**	0.514	100,1	8.26	0.083
Heterophil:Lymphocyte	100,1	2.42	0.120		100,4	15.12	**0.004**	100,1	0.01	0.924		100,1	2.72	0.099		100,1	4.79	0.309
Heterophils	100,1	12.71	**0.005**	0.589	100,4	4.59	**0.002**	100,1	0.01	0.931		100,1	8.61	**0.004**	0.026	100,1	1.99	0.103
Lymphocytes	100,1	4.59	**0.035**	-0.311	100,4	5.30	<**0.001**	100,1	1.15	0.286		100,1	11.10	**0.001**	0.014	100,1	2.23	0.072
Basophils	100,1	4.37	**0.039**	-0.520	100,4	0.48	0.747	100,1	0.93	0.337		100,1	1.27	0.263		100,1	1.30	0.278
Monocytes	100,1	0.68	0.412		100,4	0.58	0.676	100,1	0.35	0.852		100,1	0.07	0.788		100,1	0.19	0.943
Eosinophils	100,1	7.25	**0.008**	-0.505	100,4	2.80	**0.031**	100,1	9.19	**0.003**	0.576	100,1	22.57	<**0.001**	0.033	100,1	1.84	0.129
Heat Shock Protein 70	99,1	0.50	0.481		99,4	9.50	**0.049**	99,1	0.16	0.686		99,1	0.47	0.495		99,1	1.75	0.781

[†]Reference category is 'male'.

[‡]Reference category is 'control'.

[§]A derived covariate calculated per individual as follows: (individual trait value) - (sex- and season-specific trait mean).